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Original Article

The Laboratory Opossum (*Monodelphis domestica*) as a Natural Mammalian Model for Human Cancer Research

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Abstract: This study established that human cancer cells (A375 melanoma, HT-29 colon cancer, PC-3p prostate cancer) that were xenografted into suckling opossums could proliferate and globally metastasize as early as 11 days after injection. Light and electron microscopic examinations (HT-29 colon cancer) determined that the cellular features exhibited by the xenogeneic human tumors grown in laboratory opossums were consistent with those observed in tumors removed from humans. The tumor induction rate, patterns of tumor growth and regression, and types of host immune responses against the xenografted tumors were influenced by injection dosages, injection sites and injection ages of suckling opossums. The results highlight the value of the opossum model as a natural *in vivo* system for investigating human cancer growth, metastasis and apoptosis at the cellular and molecular levels; enhancing identification of tumor associated antigens or T cell epitopes through use of humoral and cellular expression cloning techniques; elucidating mechanisms utilized by tumor cells to evade host immunosurveillance; and devising diagnostic and therapeutic methods for cancer treatment.
Keywords: animal model, human cancer, Opossum, *Monodelphis domestica*

Introduction

Equipped with improved knowledge and advanced technologies, investigators are now able to make rapid progress in cancer immunology. With the successful devising and application of serological expression (SEREX) and cytotoxic T lymphocyte (CTL) based expression techniques, many human tumor associated antigens (TAAs) and T-cell specific epitopes have been identified and cloned [1, 2]. The utility of these approaches, however, has been limited by the fact that human tissues are required to conduct the experiments and that tumor cells can evade host immunosurveillance. As a result, many TAAs and T cell epitopes remain to be identified. The potential of these technologies has been further limited by lack of an animal model that can complement this deficiency. The widely used athymic nude mice are immunoincompetent. Mice that are immunologically suppressed as a consequence of treatment by immunosuppressors (e.g. cortisone, cyclosporine A) also are inappropriate because the xenografted cells also are suppressed by the same drugs [3].

Another intensively investigated topic in oncology is metastasis, which is the biggest threat to patient survival. The most widely used *in vivo* model to study metastasis is again the athymic nude mice. However, malignant tumors xenografted into the nude mice rarely metastasize spontaneously [4]. In addition, the clinical predictability of antitumor drugs screened in the nude mice is low [5].

The best animal model to simulate human cancer cell behavior would be receptive to xenografts of human cancer cells in a more natural immunological environment. Ideally, the model would initially exhibit immunodeficiency (to allow tumor establishment) but would eventually acquire immunocompetency. Another characteristic of a good model would be the capacity to support metastasis of human cells and preferably an initial micrometastasis stage. Finally, an

optimal animal model would eventually generate tumor specific markers and would show a predictable translation of model behavior into corresponding effects within humans.

The gray short-tailed laboratory opossum, *Monodelphis domestica* (family: *Didelphidae*), is a small (80-120 g), docile animal which breeds throughout the year and produces large litters [6]. Newborn opossums are at a stage approximating that of 14-day fetal rats or 40-day-old human embryos. Unlike most marsupials, *Monodelphis* females lack a pouch and neonatal pups are exposed and therefore can be easily manipulated experimentally. *Monodelphis* are predominately used as the prototype laboratory marsupial for research on normal developmental processes early in life (i.e., those that occur after birth in *Monodelphis* and before birth in rats and mice), and on experimental perturbations of those processes.

Baker *et al* [7] reported a developmental delay in the maturity of the immune system of the brushtail possum, *Trichosurus vulpecula*. Although the immune system is not fully mature until after the age of weaning, it begins to mature rapidly after 50 days of age, which is approximately equivalent to 14 days of age for *Monodelphis*. Functionally, *Monodelphis* exhibit an atypical secondary response to particulate antigens such as sheep red blood cells [8]. Peripheral blood lymphocytes respond by proliferation to Concanavalin A and other mitogens, but are stimulated weakly or not at all by allogeneic or xenogeneic (mouse) cells in mixed lymphocyte culture (MCL) [9, 10]. Despite the weak MLC response, which was not due to genetic homogeneity, allogeneic tail skin grafts were rejected promptly, suggesting that the cellular immune response of *Monodelphis* is similar to that of eutherian mammals with the exception of a weak MLC response [11].

We hypothesized that at early developmental stages, the opossum's incompetent T cell-mediated self-recognition may 1) provide an opportunity for xenogeneic tumors to establish, and 2) lead to immunotolerance to xenogeneic tumors. The use of neonatal opossums to grow allografted and xenografted (murine) melanoma cells was successful [12, 13]. In this article, we report and discuss our findings on the growth and regression patterns of xenogeneic human tumors; the influence of dosage, sites of injections, and age of animals at the time of injection; the cellular features at the light microscopic and ultrastructural levels; the host immune responses against xenografted tumors, as reflected by the histopathological features of the tumors; and the differential host immune responses against rapidly growing colon cancer cells versus dying colon cancer cells as a result of mitomycin treatment.

Materials and Methods

Experimental Animals

The animals used in this study were produced in the colony maintained at the Southwest Foundation for Biomedical Research (SFBR), San Antonio, Texas. All animals were maintained and bred as described [6].

Mothers with litters older than five days were anesthetized by conventional halothane inhalation [14]. This method, which involves putting the mother with litter in a beaker pre-equilibrated with halothane, frequently results in loss of newborn pups that release the nipples during anesthesia, or after the mother recovers from anesthesia and behaves in an agitated manner. Therefore, mothers carrying pups that were younger than five days were anesthetized by a newly devised halothane inhalation method [15]. By placing over the mother's head a 50 ml conical tube, which was pre-equilibrated with 1 ml halothane on a cotton ball, we were able to induce rapid anesthesia of the mother while sparing the babies from contact with the anesthetic.

Because opossum mothers lack a pouch, when a mother is laid on her dorsal surface, the neonates are fully exposed, each affixed to a nipple. A 29-gauge needle attached to a 0.3 ml insulin syringe was used for injection of tumor cells. After completion of the injections, the mother was returned to the original cage. The litters were observed weekly for survival and tumor growth.

Pups bearing tumors were euthanized by CO_2 inhalation for necropsy. Tumors and peripheral lymph nodes, including inguinal, suprascapular and subaxillary groups, and visceral organs were dissected and fixed in formaldehyde for pathology examination. Bodies of small pups were fixed in 10% neutral buffered formalin

Table 1 Induction, growth, metastasis and regression of human tumor cells in opossums as observed by clinical inspection and pathology

A. A375 melanoma

Litter No.	Initial litter size	Injection age (days)	Dose	No. of pups with tumor/total remaining no. of pups at the indicated weeks after injection (1-10)									
				1	2	3	4	5	6	7	8	9	10
1	12	1	0.1×10^6	0/9	0/9	0/7	0/7	2/7*	1/7	1/7†	0/0		
2	11	0	0.25×10^6	0/10	10/10	10/10	6/6	6/6*	4/6	4/6**	0/0		
3	9	0	0.25×10^6	0/5	5/5	5/5	5/5	5/5*§	2/2§	0/0			
4	8	0	0.25×10^6	0/5	5/5	5/5	5/5	5/5§	0/0				
5	10	0	0.25×10^6		3/7	7/7	7/7	6/6*	4/6†	0/0			
6	13	2	0.25×10^6	0/9	7/9	7/9	7/9†	4/6†*‡	1/3*	1/3*	0/3¶		
7	7	2	1.0×10^6	0/4	4/4	4/4	4/4	4/4*†‡	3/3*	0/3†	0/0		
8	6	6	2.0×10^6	0/6	2/6	5/5	5/5†‡	0/4*†	0/2†	0/0			

B. PC-3p prostate cancer

Litter No.	Initial litter size	Injection age (days)	Dose	No. of pups with tumor/total remaining no. of pups at the indicated weeks after injection (1-10)									
				1	2	3	4	5	6	7	8	9	10
1	11	0	0.25×10^6	0/11	2/11	4/10	7/8†‡	3/5*†‡	1/3	0/3	0/3†	0/0	
2	12	0	0.25×10^6	0/12	0/11	2/11	3/4	2/3*	0/2†	0/1	0/1	0/1	0/1 ‖
3	9	1	0.5×10^6	2/8	3/8	7/8	6/6	3/6*	2/6	0/6†	0/4¶		
4	10	2	1.0×10^6	0/9	7/7	7/7	4/4	4/4†‡	2/2*	0/2	0/2	0/2†	0/1††
5	7	3	1.0×10^6	0/4	4/4	4/4	4/4	2/4*	2/4†	0/0			
6	9	8	1.0×10^6	2/9	4/8†	4/7†	3/5	3/5†*	0/0				
7	9	3	1.5×10^6		7/7	7/7	5/5	3/4*†‡	0/0				
8	9	3	2.0×10^6		2/2	2/2†‡	0/0						
9	6	3	2.0×10^6		0/0								
10	10	7	2.0×10^6	3/10	10/10	10/10	10/10	10/10	10/10*	2/10	0/10¶		

C. HT-29 colon cancer

Litter No.	Initial litter size	Injection age (days)	Dose	No. of pups with tumor/total remaining no. of pups at the indicated weeks after injection (1-10)									
				1	2	3	4	5	6	7	8	9	10
1	11	0	0.25×10^6	0/7	0/7	0/7	0/7	1/7	1/7*	0/7	0/7	0/7†	0/0
2	9	1	0.25×10^6	0/9	0/8	1/8	3/8	3/8*	0/8†	0/0			
3	10	2	0.25×10^6	0/10	0/10	2/10	2/10*§	0/7	0/7†	0/0			
4	8	2	0.5×10^6		5/5	5/5	5/5	5/5*	3/5	0/5	0/5†	0/3†	0/0
5	11	8	0.5×10^6	0/11	0/11	0/10	0/10†	0/5¶					
6	12	10	0.5×10^6	0/12	0/12	0/11	0/11†	0/6¶					
7	7	0	1.0×10^6		4/4	4/4†‡	0/0						
8	9	0	1.0×10^6			9/9	9/9	5/9*	5/9†	0/4	0/4†	0/0	
9	7	1	1.0×10^6			5/5	5/5	5/5*†‡	0/0				
10	10	2	1.0×10^6		2/3	2/3	2/3	2/3*	2/3†	0/0			
11	9	3	1.0×10^6	4/6	4/6†	0/0							
12	9	3	1.0×10^6	5/6	5/6	5/6	5/6*†	0/0					
13	8	5	1.0×10^6		3/7†‡	2/6†	0/3*	0/3†	0/2†	0/0			
14	8	1	2.0×10^6	0/0									
15	8	2	2.0×10^6	8/8	0/0								
16	9	9	2.0×10^6	0/9	0/8	0/8	1/8	1/8†	0/4¶				
17	8	6	2.5×10^6	0/8	0/8	3/8†	2/5	0/5*	0/5†	0/3¶			
18	9	9	2.5×10^6		0/8	0/8	2/8†	0/5*	0/5†	0/0			
19	5	20	3×10^6	1/5†	3/4*	2/4	2/4	2/4*	0/4	0/4†	0/0		

* Tumor regression had occurred. † Necropsy was conducted. ‡ Metastasis had occurred. § Reduction in number was due to cannibalization by agitated mother due to light halothane anesthesia or by forced detachment of suckling pups for photographing and tumor growth monitoring. ¶ The remaining pups were used for other research purposes. ‖ Animal died at the age of 23 months. ** Death from unknown causes of mother and litter. ††Animal is still alive.

The dose as stated does not necessarily denote the actual dose retained by the pups because a fraction of the injected cells could have been lost by postinjection leakage at the injection site, spontaneously or as a consequence of licking by the mother.

after their cranial, thoracic and abdominal cavities were opened. For electron microscopic examinations, the tumor was cut into 3 mm slices immediately after dissection and fixed in 3% glutaradehyde at 4°C.

All animals at the SFBR received humane care in compliance with the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85- 23, revised 1996). Prior to initiating this study, the SFBR Institutional Animal Care and Use Committee reviewed and approved the protocol related to this study.

Cell Lines

The human A375 melanoma cells and PC-3p prostate cancer cells were obtained from the University of Texas M.D. Anderson Cancer Center and were cultured in a humidified 5% CO_2/95% air incubator. The A375 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and the PC-3p cells were cultured in Ham's F12K medium containing 10% fetal bovine serum, respectively.

The human HT-29 colon cancer cells used in this study were purchased from American Type Culture Collection (ATCC) and cultured in McCoy's 5a medium supplemented with 10% fetal bovine serum in a humidified 5% CO_2/95% air incubator. HT-29 cells grow as colonies in culture. Because HT-29 cell counts using a hemocytometer were not accurate due to cell clumping, the number of cells injected was estimated by assuming that HT-29 and A375 cells have the same size and comparing the pellet sizes of the HT-29 cells and A375 cells, which do not clump and can be accurately counted. Because the body temperature of adult opossums is 32.6°C, all cells were cultured at 33°C.

For the growth inhibition experiment, the HT-29 cells that were cultured to confluence were treated with 1 mg/ml mitomycin (Sigma Inc.) for 24 hr. The mitomycin-treated HT-29 cells and untreated HT-29 cells were harvested at the same time, and injected in the same doses to the same animals at different sites (lower dorsal sides). All three cell lines grow well at 33°C. The cells were cultured to confluence in 100 × 20 mm culture dishes before being harvested for injection. Harvested cells were washed two times with 1 X phosphate buffered saline (PBS) and resuspended in a volume of PBS equal to the volume of the pellets for injection.

Results

Establishment, Growth, Metastasis and Pathological Features of Xenografted Tumors

As shown in **Table 1**, 335 neonatal suckling opossums of different ages and belonging to 37 litters were injected *s.c.* into the dorsal side with different doses of human A375 melanoma cells, PC-3p prostate cancer cells, or HT-29 colon cancer cells.

A375 melanoma

The dosage of 0.1×10^6 A375 melanoma cells injected into 12 1-day-old (1-d.o.) pups of litter 1 (**Table 1A**) was inadequate to consistently induce tumors for at least five weeks after injection although two had observable tumors which quickly regressed. Necropsies at week 7 showed that one pup had a 0.5 x 0.75 cm *s.c.* tissue mass, but all others were negative.

When we injected 0.25×10^6 A375 cells into 51 0-2-d.o. pups belonging to five litters (litters 2-6, **Table 1A**), a 100% tumor-take rate was observed among the 27 surviving pups (litters 2-5) that were injected at 0 days old. Average tumor size was approximately 0.5 x 0.75 cm by week 4, and the tumors started to regress by week 5. In comparison, when the same dose was injected into 13 2 d.o. pups (litter 6), the tumor-take rate was 78% by week 2 (**Figure 1A**); by week 4, one larger tumor had grown to 1.25 x 1.5 cm; by week 5, the tumors started to regress (**Figure 1B**).

Necropsies of the six pups of litter 5 (**Table 1A**) at week 6 showed that one pup had tumor invasion into the spinal column, which caused bony distortion of the pup's body; three pups exhibited regressing *s.c.* tumors, measuring 0.25 x 0.25 cm; two other pups were negative. Necropsies of six tumor-bearing pups (litter 6) at weeks 4 and 5 showed that while the 4-week-old (4-w.o.) tumors looked newly established, the 5-w.o. tumors showed signs of regression, i.e., whitish dots and streaks scattered on the surface of the tumors (**Figure 1B**). Upon further examination, one of the three pups exhibited a large solid fresh tumor,

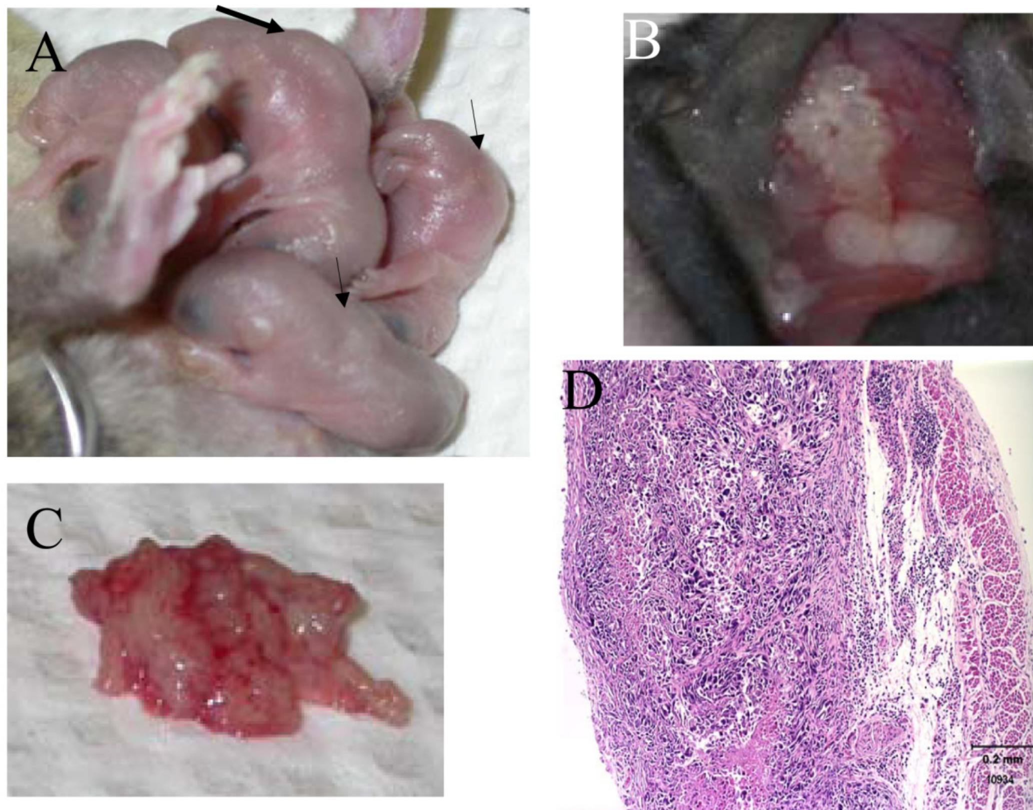

Figure 1 Human melanoma cells xenografted into suckling young opossums. A. Suckling pups at two weeks of age (litter 6, Table 1A). Amelanocytic tumors, as pointed by arrows, were induced by *s.c.* injection of 0.25 × 10^6 A375 cells at the age of 2 days. B. Regressing xenografted human melanoma at week 5 after injection (litter 6). Whitish dots and streaks were evident in the tumors. C. A large solid tumor located in the muscular tissues of chest wall in a pup of litter 6. This 5-w.o. tumor appeared to have been newly established by comparison to the regressing *s.c.* tumor shown in B. D. Tumor of 4-w.o. *Monodelphis* (litter 6). Proliferation of tumor cells was associated with considerable cell death and mineralization. Minimal host inflammatory cells were noted. Hematoxylin and eosin (HE), bar = 0.2 mm.

measuring 1.25 x 1.25 cm, in the left chest wall (Figure 1C).

Microscopic examination of the six necropsied pups of litter 6 revealed that tumor cell proliferation associated with considerable cell death and mineralization in the 4-w.o. tumor (Figure 1D). Inflammatory responses, however, were not remarkable. Findings on the 5-w.o. tumors were similar, except for moderate host inflammatory reactions. Metastasis was detected in two 5-w.o. pups, one was found in the meninges and the other was found in the lungs. Pathologic examination of a 6-w.o. tumor-bearing pup (litter 5) did not reveal any metastatic foci.

After 1.0×10^6 cells were injected into seven 2-d.o. pups (litter 7), four survived. Tumors, measuring 0.25 x 0.5 cm, became observable during week 2, and continued to grow to 0.5 x 1.0 cm during two more weeks before regression occurred at week 5. Necropsy of one pup at week 5 exhibited a regressing 0.5 x 0.5 cm *s.c.* tumor mass and exhibited multifocal lung metastasis. Necropsies of the other three pups that carried regressed tumors at week 7 exhibited no positive findings.

Pups at 6 days old (litter 8) tolerated 2.0 × 10^6 cells. The average tumor size was 0.25 x 0.25 cm at week 3 and grew to 0.5 x 0.5 cm by week 5. In contrast to the 4-w.o. tumor of litter 6 (see above), pathology of one pup at week 4 showed good tumor cell growth with moderate primary neutrophilic inflammation. The lung contained a single inflammatory neutrophilic focus.

PC-3p Prostate Cancer

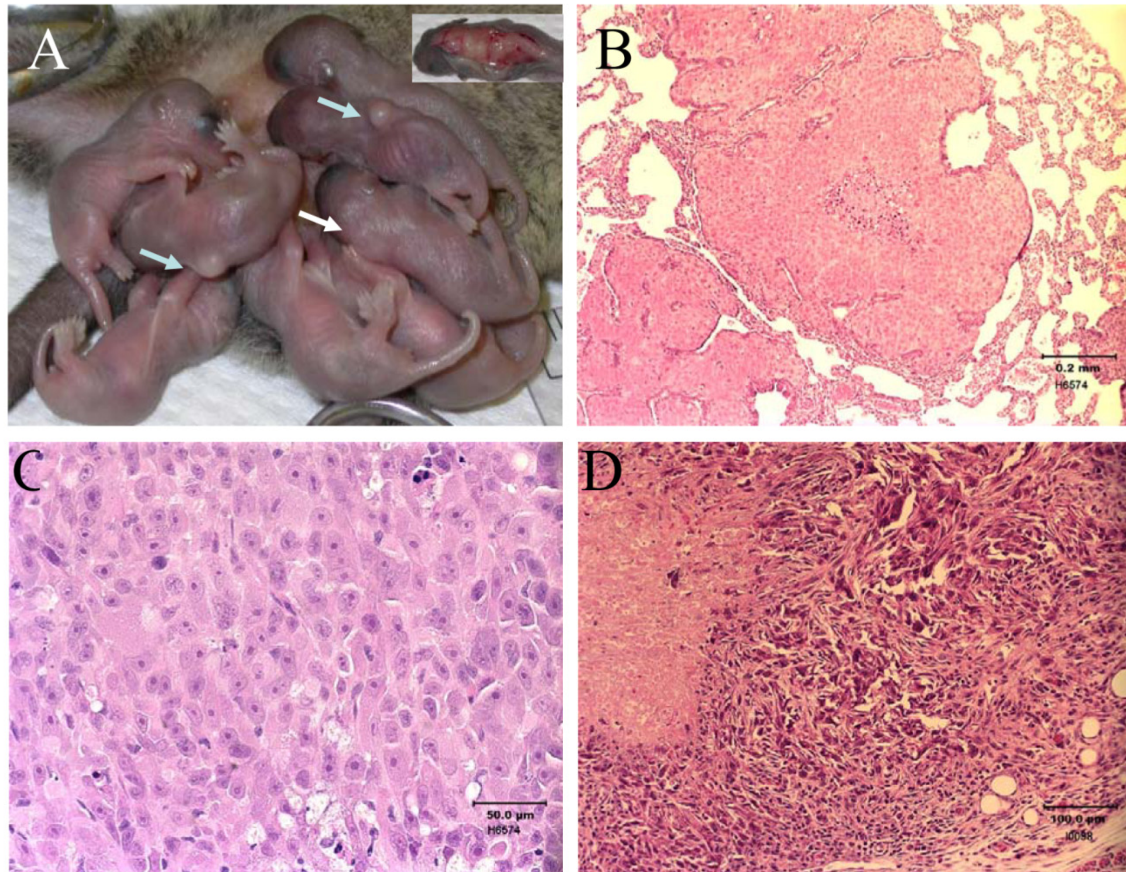

Figure 2 Human prostate cancer cells xenografted into suckling opossums. **A.** Suckling opossums at 3 weeks old (litter 1, **Table 1B**). The tumors (arrows) were induced by *s.c.* injection of 0.25 × 10^6 PC-3p cells on the day of birth (0-d.o.). During week 4, one pup of litter 1 (white arrow) appeared sick and was euthanized (window). Two fresh-looking tumors exhibiting aggressive growth were observed at necropsy. **B.** Lung tissue with multifocal metastatic prostate cancer from the same pup as shown in the window of **A**. Prominent central necrosis was associated with some proliferative foci. HE, bar = 0.2 mm. **C.** Prostate cancer of a 5-w.o. pup (litter 1). Tumors cells are proliferative and there is no indication of host rejection. HE, bar = 50 µm. **D.** Prostate cancer induced by *s.c.* injection of 2 × 10^6 PC-3p cells into a 3-d.o. pup (litter 8). In contrast to the proliferative and viable 5-w.o. tumor shown in **C**, this 3-w.o. tumor exhibited resorption and death of the tumor cells. HE, bar = 100 µm.

The 0-d.o. pups that were injected with 0.25×10^6 PC-3p cells (litters 1-2, **Table 1B**) exhibited observable tumors during week 2; by week 3, the average tumor size was 0.5 x 0.5 cm (**Figure 2A**). During week 4, some tumors grew to 0.75 x 0.75 cm and one pup of litter 1 (**Table 1B**) appeared sick and was euthanized (**Figure 2A**, pop-up window). Necropsy exhibited two fresh-looking tumors. One measured 0.5 x 0.5 cm. The other, which measured 1.0 x 1.0 cm, exhibited aggressive growth. It occupied the entire left chest wall, which compressed the chest cavity. Pathological examination of this pup revealed viable neoplastic cells with local invasion into soft tissues behind the skull and in skeletal muscles of the forearm with extension into the thoracic cavity, pericardial sac, paravertebral thoracic skeletal muscle and meninges. Metastatic tumor foci were evident throughout the lungs. Some of these proliferative foci had prominent central necrosis (**Figure 2B**). By week 5 (litter 1), aggressive tumor growth was still observed in a pup of litter 1, showing no signs of host rejection (**Figure 2C**). Metastases to the meninges and lungs were also detected in this pup. Two pups died during each of week 4 and week 5 and necropsies of the three 8-w.o. pups exhibited no positive findings (litter 1, **Table 1B**). One pup in litter 2 is still alive.

When 0.5 × 10^6 PC-3p cells were injected into nine 1-d.o. pups (litter 3), tumors were induced in all six surviving pups by week 4, measuring

0.5 x 0.75 cm. Tumors started to regress during week 5. Necropsy of two pups at week 7 exhibited regressed *s.c*. tumor tissue streaks.

The dosage of 1 × 10^6 PC-3p cells resulted in a 100% tumor-take rate when injected into 2 3-d.o. pups (litters 4-5, Table 1B), but only 50% in the 8-d.o. pups (litter 6). The average tumor size was 0.5 x 0.75 cm by week 4, with the largest measuring 1.0 x 1.5 cm. Tumor regression was observed at week 5 and after. Necropsy of two pups of litter 4 at week 5 revealed a 1.0 x 1.5 cm s.c. tumor, which appeared newly established; necropsy of a third pup at week 9 exhibited a completely regressed *s.c.* tumor. The fourth pup (litter 4) died at the age of 23 months. Pathology of a 5-w.o. pup (litter 4) revealed that the tumor exhibited marked growth and expansion both through the skin with ulceration and into the deep skeletal muscle as well as the sternum, and with penetration into the chest cavity. The minimal inflammatory infiltrates in this tumor were essentially all lymphocytic. Metastatic sites were seen in both kidney and liver of this pup. Pathology of a 6-w.o. pup in litter 5 revealed granulomatous reaction and no viable prostate cancer cells.

Similarly, 1.5 × 10^6 cells injected into nine 3-d.o. pups (litter 7) also led to a 100% tumor-take rate. The tumor growth and regression patterns were similar to those of litters 4-6 (Table 1B). In contrast to the proliferative 5-w.o. tumors induced by 1.0 × 10^6 cells (litter 4), pathology of two of the four necropsied 5-w.o. pups (litter 7) showed that the tumor cells were essentially being destroyed, with only a few viable cells seen. Metastasis to the meninges, lungs and kidneys were detected in these animals.

The dosage of 2 × 10^6 PC-3p cells was poorly tolerated by 3-d.o. pups (litters 8-9, Table 1B): only two of the 11 injected pups survived more than two weeks. Necropsy of the two surviving pups at week 3 (litter 8) revealed 0.5 x 1.0 cm *s.c.* tumors that appeared to have been newly established. However, the relatively large tumor burden impacted the viability of tumor cells: pathology of two 3-w.o. tumor samples (litter 8, Table 1B) revealed resorption and death of the tumor cells at the original injection sites with meningeal metastasis in one pup (Figure 2D).

The same dosage was well tolerated by 7-d.o. pups (litter 10, Table 1B), and the tumor-take rate was 100%. Tumors measured about 0.25 x 0.5 cm by week 2; by week 4, the average tumor size had more than doubled, with the largest measuring 1.5 x 1.5 cm. Tumors started regressing remarkably during week 6; by week 8, the tumors had become non-observable.

HT-29 Colon Cancer

For the 30 pups (litter 1-3, Table 1C) injected by 0.25 × 10^6 HT-29 cells, six pups developed observable tumors by week 5. The tumor size was relatively small, measuring about 0.25 × 0.25 cm.

An increased dosage of 0.5 × 10^6 cells induced tumors in all five surviving pups of litter 4, which grew to 0.5 × 0.75 cm before starting to shrink by week 5 (Figure 3A). This dosage, however, did not induce tumors in 8-10-d.o. pups (litters 5-6).

The dosage of 1.0 × 10^6 cells was injected into the pups of 7 litters (litters 7-13) consisting of 59 pups of 0-5 days of age. Tumors were analyzed at necropsy at ages 2, 3, 4, 5, 6 and 8 weeks. Necropsy of six 2-w.o. pups (litter 11) and four 3-w.o. pups (litter 7) exhibited well-established *s.c.* tumors (Figure 3B). Necropsy of six 4-w.o. pups (litter 12) at week 4 and five 5-w.o. pups (litter 9) exhibited tumors that were intermixed with regressing whitish tumor streaks (Figure 3C). In comparison, necropsy of the nine 6-8-w.o. pups (litter 8) showed regressed tumors. It is noteworthy that, like the A375 melanoma (Figure 1C), one 5-w.o. pup (litter 9) carried two contrasting tumor foci: one regressed *s.c.* tumor and another fresh-looking intramuscular (chest wall) tumor, which measured 0.5 × 1.25 cm (Figure 3D).

Histologically, 2-w.o. tumor cells were extremely viable in appearance, and there were no infiltrations of host inflammatory cells (Figure 3E). At week 3, host inflammatory responses started to appear (Figure 2F). At week 4, pyogranulomatous formation and necrosis of tumor cells were seen, but a few viable tumor cells were still present. By week 5, the pyogranulomatous responses and tumor cell death were more remarkable. Finally, the 6-w.o. tumor was dissected into numerous granulomas, indicating that the tumor was undergoing resolution by the host.

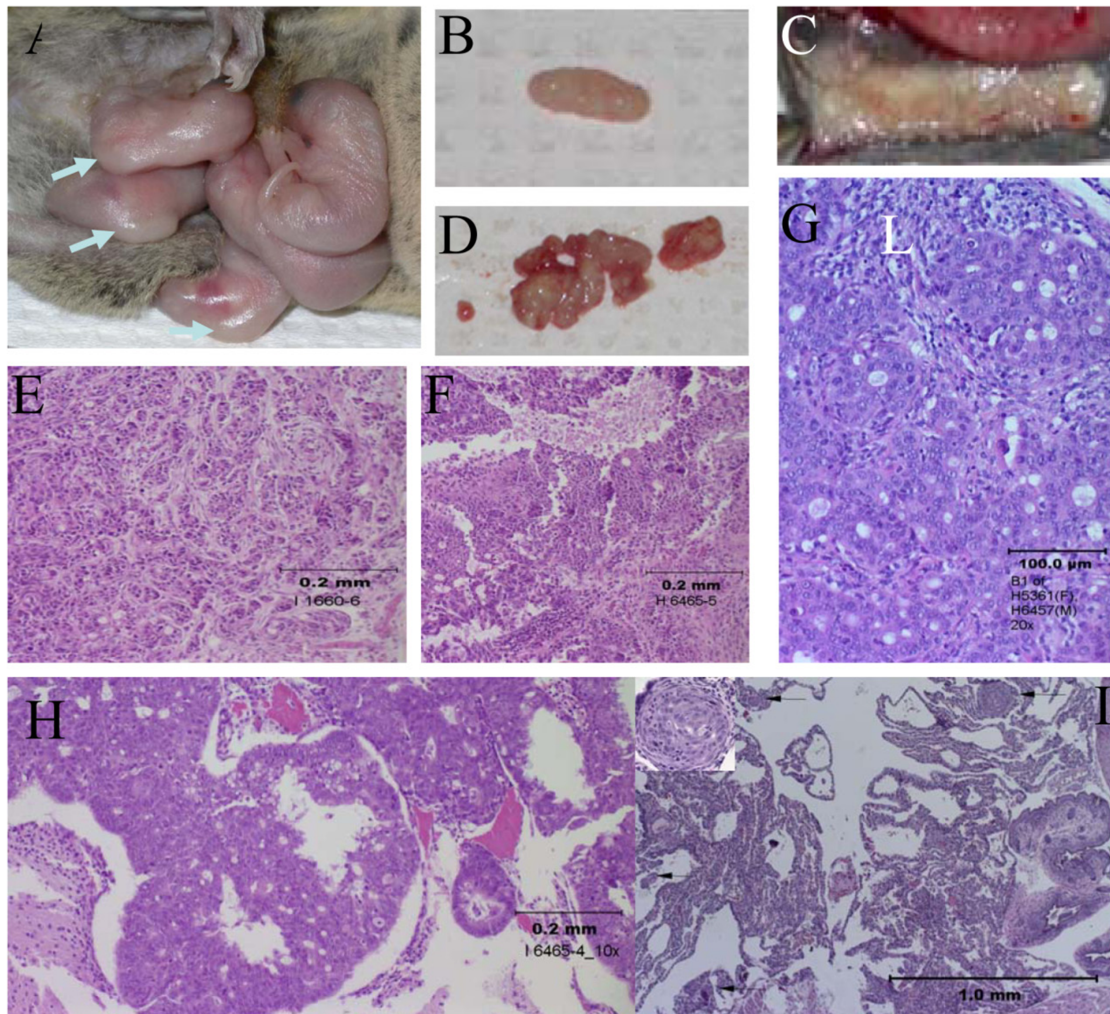

Figure 3 Human colon cancer cells xenografted into suckling opossums. A. Suckling pups at 2-w.o. (litter 4). All five pups carried *s.c.* tumors (arrows) induced by *s.c.* injection of 0.25 × 10^6 HT-29 colon cancer cells into 2-d.o. pups. B: HT-29 colon cancer established two weeks after injection of 1.0 × 10^6 cells into a 0-d.o. pup (litter 7). The *s.c.* tumor appeared to be well-established with no signs of regression. C: The *s.c.* tumor established five weeks after injection of 1.0 × 10^6 cells into a 1-d.o. neonate (litter 9). The tumor was intermixed with regressing whitish tumor streaks. D: The intramuscular (chest wall) tumor found in the same pup as in C. On gross examination, the tumor looked newly established in comparison with the regressing *s.c.* tumor. E. A 2-w.o. tumor established in a 3-d.o. pup (litter 11) by *s.c.* injection of 1.0 × 10^6 cells. There was essentially no lymphocytic or other cellular response to the 2-w.o. tumor. HE, bar = 0.2 mm. F. A 3-w.o. tumor established in a 0-d.o. pup (litter 7) by *s.c.* injection of 1.0 × 10^6 cells. A granulomatous response and lymphocytic infiltration were detected in this tumor sample. HE, bar = 0.2 mm. G. A 4-w.o. tumor established in a 9-d.o. pup (litter 18) by *s.c.* injection of 2.5 × 10^6 cells. There was a moderate inflammatory infiltrate associated with this neoplasm, which, in contrast to tumors induced by injection of neonatal pups, was primarily lymphocytic (L). Additionally, it was supported by moderate interstitial fibrosis and neovascularization. HE, bar = 0.1 mm. H. A 3-w.o. tumor that had metastasized to the meninges of a pup injected at 0 days of age with 1.0 × 10^6 cells (litter 7). Neoplastic cells were evident in the vessels of meninges. No immune response was detected. HE, bar = 0.2 mm. I. Multifocal embolic metastatic cells in the septa of lungs (arrows) of a pup of litter 13 that were necropsied at the eleventh day after injection. The window in the upper left corner shows one of the metastatic foci at a higher magnification. HE, bar = 1.0 mm.

It is noteworthy that pathological examination of one 4-w.o. tumor sample (litter 18, Table 1C) induced by injection with 2 × 10^6 cells into a 9-d.o. pup showed that the tumor was expansile, invasive and had a moderate mitotic rate. There was a moderate inflammatory infiltrate associated with this neoplasm, which was primarily lymphocytic (Figure 3G). Additionally,

it was supported by moderate interstitial fibrosis and neovascularization.

To assess metastasis, systemic pathology was carried out on four pups with 3-w.o. tumors (litter 7), one pup carrying a 5-w.o. intramuscular tumor in the chest wall (litter 9), and five pups with 6-w.o. tumors (litter 8). The results showed that 1) in the four pups of litter 7, neoplastic cells were detected within the meninges of the brain and spinal column in one of the four pups (**Figure 3H**) and no metastasis was detected in the other three pups; 2) meningeal adenocarcinoma was observed in the pup carrying the intramuscular tumor in the chest wall (litter 9); 3) no metastasis was detected in the five pups of litter 8. It is noteworthy that one pup of litter 13 appeared very sick by the 11th day after injection and was euthanized. Pathological examination showed multifocal metastatic cells in the lungs and abdominal cavity (**Figure 3I**).

The dosage of 2.0×10^6 cells was excessive for 1-2-d.o. opossums (litters 14-15), but was well tolerated by 9-d.o. pups (litter 16). The tumor-take rate, however, was only 13% for this litter (1 of 8 surviving pups).

An experimental dosage of greater than 2×10^6 cells injected into 35 pups belonging to litters 17-19. Although only two pups of litter 17 carried observable tumors by week 3, necropsy of three non-tumor bearing pups of this litter showed that one was positive. In comparison, when the same dosage was injected into eight 9-d.o. pups (litter 18), two pups exhibited tumors by week 4. Necropsies of these two pups and one littermate at that time exhibited *s.c.* tumors, measuring 0.25 × 0.25 cm in one pup and 0.75 × 1.0 cm in another. There was no positive finding in the third pup. Necropsies of the other five pups during week 6 were unremarkable. Tumors started to regress by week 5 after injection.

When 3×10^6 cells were injected into five 20-d.o. pups, no tumors were observed one week after injection. Necropsy of one pup at this time, however, revealed tumor induction. By week 2, tumors were observable in three of four remaining pups; one had regressed during the following week; the other two persisted until week 5 after injection.

Ultrastructural Cellular Features of Xenografted Human Colon Cancer

To determine if the xenogeneic tumors grown in opossums can exhibit cellular features that are consistent with the human condition, we studied a 3-w.o. tumor, measuring 0.75 x 0.75 cm, from a pup that was injected with 1×10^6 HT-29 cells at the age of 3 days. Using electron microscopy, we examined a number of ultrastructural cellular features, which included morphology of nuclei and nucleoli, mitochondria, junctions, lumens, microvilli, and secretory granules.

Ultrastructural evaluation revealed neoplastic cells that showed clusters and glandular-like formations. Morphologically, the cells varied from oval to columnar with large nuclei and distinct nucleoli. Nuclei varied from round to oval with irregular to cleaved margins. Nucleoli were prominent and, in scattered nuclei, were in doublets; mitoses were present. Mitochondria were present and varied from scattered clusters (most cells) to completely filling the cytoplasm (rare cells) (**Figure 4A**). Junctional complexes were prominent (**Figure 4A**). The cytoplasm of the cells contained ribosomes, glycogen, and to lesser extent intermediate filaments (**Figure 4A**). Scattered mitotic spindles were present (**Figure 4B**). Microvilli were present and tended to show apical orientation in areas with formed lumina (**Figure 4C**). These findings were consistent with an adenocarcinoma.

Host Immune Response against Vital versus Dying Colon Cancer Cells

Because the laboratory opossum is capable of rejecting allografted skin tissue [11], it is reasonable to assume that regression of the xenografted tumors is a result of natural rejection of foreign tissues. However, it remains to be determined whether active tumor growth, which leads to differential expression of TAAs at different tumor progression stages [16], also contributes to rejection.

We prepared two sets of HT-29 cells: one was treated with mitomycin and one was untreated. Treatment with mitomycin drastically changed the growth pattern of the HT-29 cells. Growth of the mitomycin-treated cells was slowed, but not stalled, because the cells were still able to grow by forming large colonies for about one week. Then growth of the cells stopped, and

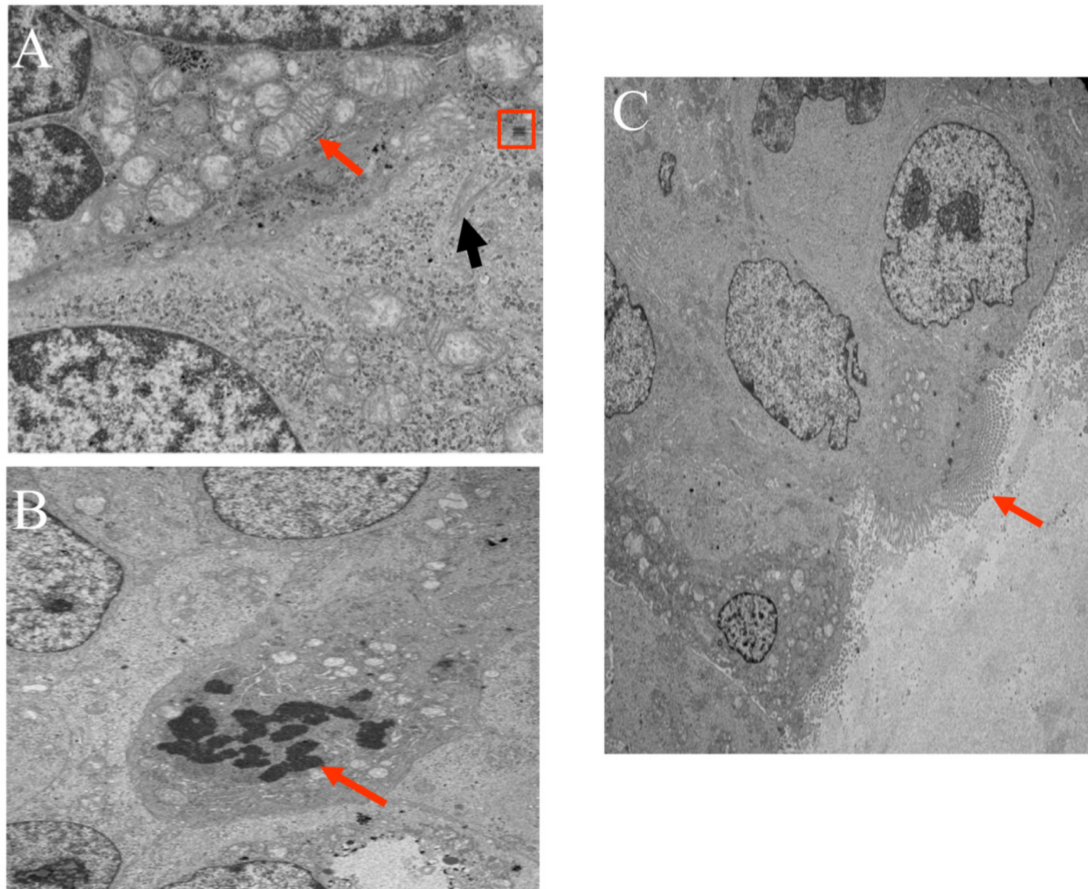

Figure 4 Ultrastructural cellular features of xenografted 3-w.o. colon cancer cells injected with 1 x 10^6 HT-29 cells at the age of 3 days. **A.** Mitochondria are present in scattered clusters (arrow), and junctional complexes are prominent (square). Black arrow is pointing to intermediate filaments. **B.** Arrow is pointing to a mitotic spindle. **C.** Microvilli are present and tend to show apical orientation in areas with formed lumen.

the cells started to detach from the culture dishes (Figure 5A).

We injected two 30-d.o. pups and four 41-d.o. pups with treated and untreated cells at a dose of 5 × 10^6 cells. At these ages, the inflammatory responses of the host against the treated and non-treated xenografted cells did not exhibit any obvious differences.

Then we injected three pups at the age of 51 days. One week after injection, we euthanized one pup, which by inspection, showed a reddish inflammatory sign at the site of injection of the untreated HT-29 cells. Upon necropsy, we found that the untreated HT-29 cells elicited a stronger inflammatory reaction from the host than the treated HT-29 cells. When we euthanized the other two pups four days later, we found that one pup showed a similar response, i.e., the untreated cells were more inflammatory than the treated cells. However, the other pup showed no difference between the sites injected with treated and untreated cells, and thus the inflammation in this pup was self-resolving.

We proceeded to inject seven 57-d.o. pups. One week after injection, we euthanized five pups and found that, similar to the 51-day-old pups, the untreated HT-29 cells elicited a much stronger inflammatory reaction from the host than the treated HT-29 cells (Figure 5B). However, by the time of necropsy of the remaining two pups at two weeks after injection, the inflammatory responses were resolved and no differences between the two injection sites were detectable.

Discussion

Host tolerance to grafted foreign tissue

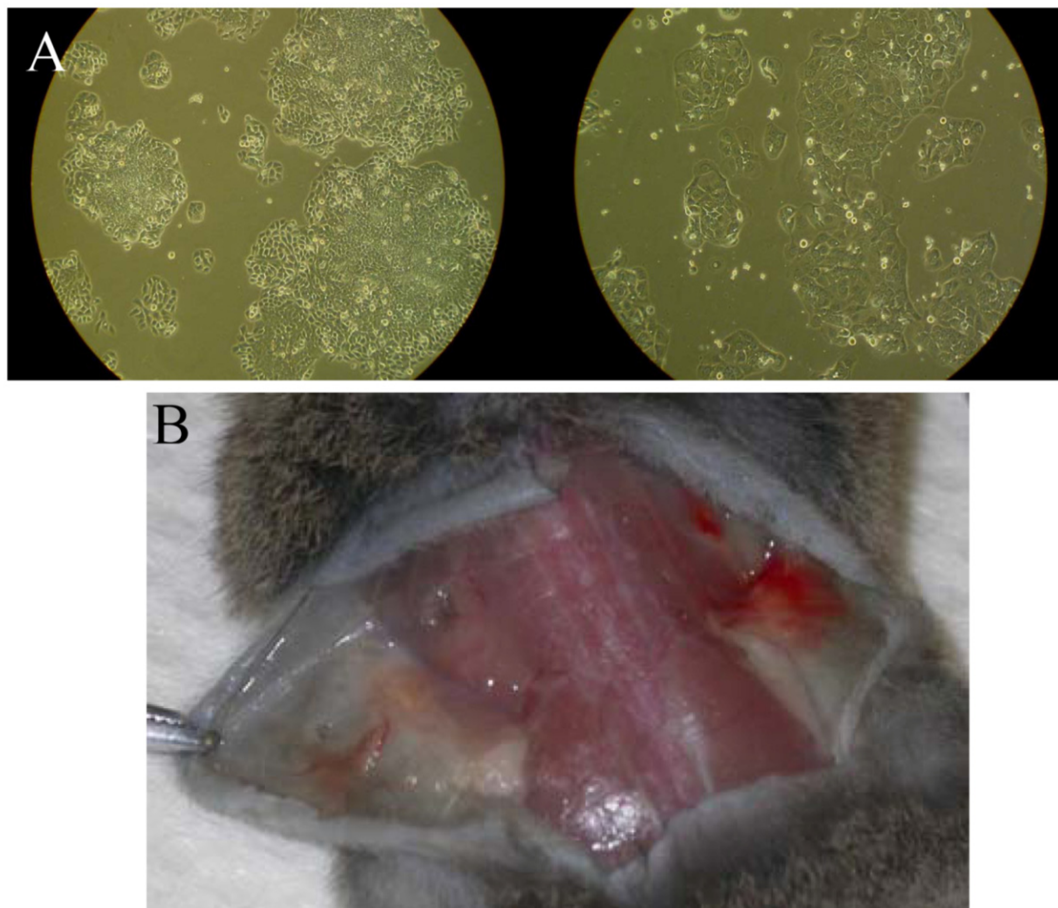

Figure 5 Host immune response against viable vs. dying colon cancers. **A.** HT-29 colon cancer cells (left) and HT-29 cells at one week after mitomycin treatment (see text for details). Mitomycin-treated HT-29 cells were still able to attach and form colonies. However, the growth of the mitomycin-treated cells was stalled one week after treatment and the cells then started to detach. **B.** A 57-d.o. opossum injected with HT-29 colon cancer cells and mitomycin-treated HT-29 colon cancer cells and necropsied one week later. The untreated HT-29 cells elicited greater inflammatory reaction (right lower dorsal region; reddish inflammation can been seen) than the treated HT-29 cells (left lower dorsal region).

remains a complicated topic in immunology. It is well-known that down-regulated MHC-1 expression in cancer cells plays a role in the induction of host tolerance [17], and this characteristic of cancer cells probably contributed to the results of this study. However, the delayed developmental window period, as well as other characteristics displayed by the marsupial immune system [7, 8, 9, 10, 11], probably made a more important contribution to the induction of tolerance in this study. The extrapolation from results obtained with brushtail possums [7] that immunological incompetence is most pronounced in the first two weeks of *Monodelphis* life is consistent with results from this study. It also is consistent with results from other related studies, e.g., UV-induced opossum melanoma cells allografted to opossums less than two weeks old display a capacity of aggressive tumor growth and metastasis despite MHC-1 mismatch [12], and murine B16 melanoma cells xenografted within the first two weeks of life remain viable through to the adulthood [13]. Studies using non-tumor tissues also support this finding: murine neural progenitor cells, whose MHC-1 expression is also down-regulated [18], have been xenografted into the eyes of suckling young opossums (5-10 days of age) and, unlike in opossums of more advanced age (>35 days of age), became established without eliciting any host immunological responses [19, 20].

Despite tolerance to early grafted tumor tissues, regression of xenografted tumor tissues eventually took place as the opossum's immune system matured, indicating the roles of aberrant expression of TAAs during growth of cancer cells at different stages [16, 21]. The differential host immune responses against different tumor types (e.g., prominent inflammation against colon cancer, moderate inflammation against melanoma, and minimal inflammation against prostate cancer), and against non-mitomycin treated colon cancer cells vs. mitomycin treated cells, provide evidence supporting this hypothesis.

A host immune response against cancer cells involves the interaction of many different cell types and cell products, including granulocytes [22], monocytes/macrophages [23], natural killer (NK) cells [24], dendritic cells (DCs) [25], and cytotoxic lymphocytes (CTLs) [26]. In addition to the obvious presence of granulocytes, lymphocytes and macrophages within the tumor tissues detected by light microscopic examinations, the presence of DCs and NKs infiltrating the xenogeneic colon cancer was also indicated by electron microscopic studies (unpublished data). It is noteworthy that the HT-29 cells xenografted at later ages could escape the host's innate immune attack; the tumors became observable at a much later time after injection. The histopathology results of these tumors showed predominant lymphocytic infiltration, indicating that detection and destruction of these tumors is accomplished by a different mechanism. Because CTLs constitute one of the most important effector mechanisms of antitumor immunity, the opossum model could be the only natural mammalian model for investigating the use of CTLs to combat human cancer.

We do not know if the pup from litter 9 that exhibited a regressed *s.c.* tumor, but had a fresh-looking intramuscular tumor, had cells inadvertently injected into both sites, or if the intramuscular tumor was a metastasis. In either case, the results suggest that *s.c.* tumors exhibit different growth patterns by comparison with intramuscular tumors because of different distributions of immune cells, e.g., the distribution of DCs in the *s.c.* and muscular tissue [27].

It is noteworthy that the *s.c.* xenografted HT-29 colon cancer exhibited a benign pattern of growth in most opossums, metastasis was detected in the central nervous system of only two opossum pups despite the relative large size of the established tumors, and in the lungs and abdominal cavity of one pup. Nevertheless, the relatively short time period for the HT-29 cells to spontaneously metastasize (within two weeks after injection) makes the opossum an ideal *in vivo* model for investigating metastasis. By comparison, the PC-3p prostate cancer cells and A375 melanoma cells demonstrated much more aggressive growth and a much higher rate of spontaneous metastasis: six out of seven systemically examined prostate cancer-bearing pups had metastases; four out of nine melanoma-bearing pups had metastases. These results highlight the value of the opossum model as an *in vivo* model system to investigate metastasis.

In summation, the establishment of human cancers in *Monodelphis* and the demonstration of metastatic phenotypes in a relatively short period of time indicate that *Monodelphis* will complement the murine model as a valuable resource for cancer related research. The distinct regression patterns consequent to the innate immune response and the cellular immune response of the host establish the opossum model as a novel natural system for tumor immunobiological research. Further development of this model system could lead to identification of immunological components in recognizing and combating cancer cells and devising diagnostic and therapeutic methods for cancer treatment.

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